

The chemokine Bv8/prokineticin 2 is up-regulated in inflammatory granulocytes and modulates inflammatory pain

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Neutrophil migration into injured tissues is invariably accompanied by pain. Bv8/prokineticin 2 (PK2), a chemokine characterized by a unique structural motif comprising five disulfide bonds, is highly expressed in inflamed tissues associated to infiltrating cells. Here, we demonstrate the fundamental role of granulocyte-derived PK2 (GrPK2) in initiating inflammatory pain and driving peripheral sensitization. In animal models of complete Freund's adjuvant-induced paw inflammation the development and duration of pain temporally correlated with the expression levels of PK2 in the inflamed sites. Such an increase in PK2 mRNA depends mainly on a marked up-regulation of PK2 gene transcription in granulocytes. A substantially lower up-regulation was also detected in macrophages. From a pool of peritoneal granulocytes, elicited in rats by oyster glycogen, we purified the GrPK2 protein, which displayed high affinity for the prokineticin receptors (PKRs) and, when injected into the rat paw, induced hypersensitivity to noxious stimuli as the amphibian prokineticin Bv8 did. Mice lacking PKR1 or PKR2 developed significantly less inflammation-induced hyperalgesia in comparison with WT mice, confirming the involvement of both PKRs in inflammatory pain. The inflammation-induced up-regulation of PK2 was significantly less in *pkr1* null mice than in WT and *pkr2* null mice, demonstrating a role of PKR1 in setting PK2 levels during inflammation. Pretreatment with a nonpeptide PKR antagonist, which preferentially binds PKR1, dose-dependently reduced and eventually abolished both prokineticin-induced hypernociception and inflammatory hyperalgesia. Inhibiting PK2 formation or antagonizing PKRs may represent another therapeutic approach for controlling inflammatory pain.

hypernociception | inflammation | prokineticin receptors

In past years, several groups have generated convincing evidence that neutrophil-associated hyperalgesia results from the neutrophil release of proinflammatory pronociceptive factors, including products of arachidonic acid metabolism (1, 2) and cytokines such as IL-1 β , IL-8, IL-12, TNF- α , and macrophage inflammatory proteins MIP-1a and MIP-1b (3, 4). Belonging to the chemokine family, the recently discovered prokineticins [Bv8, MIT, prokineticin 1 (PK1), and prokineticin 2 (PK2)] (5–9) activate two G protein-linked PK receptors (PKR1 and PKR2) (10–12) localized in the brain, dorsal root ganglia (DRG) neurons, granulocytes, macrophages, and endothelial cells. Bv8 was initially identified in the skin secretion of the frog *Bombina variegata*, as a secretory protein that induced hyperalgesia and gastrointestinal motility. Later on, the human orthologues of this highly conserved protein (PK1 or EG-VEGF and PK2 or mammalian Bv8) were shown to promote angiogenesis and modulate neurogenesis, circadian rhythms, hematopoiesis, and immune response (13). Activation of PKRs in primary afferent C fibers sensitizes nociceptors to thermal, mechanical, and chemical stimuli (14–16). In neutrophils, macrophages, and dendritic cells it promotes chemotaxis and cytokine release

(17–20), and in capillary endothelial cells it stimulates angiogenesis (21). Testis, peripheral blood leukocytes, and macrophages express two mRNA transcripts of PK2, one coding for the canonical PK2 and the other for an elongated form containing additional 21 basic amino acids between Lys-47 and Val-48 of the mature PK2 protein, named PK2L (6, 19, 22). In inflamed tissues both PK2 and PK2L mRNA transcripts are overexpressed (15), but the mature proteins have not yet been isolated from inflammatory cells nor tested for their biological activity.

The goal of the present experiments is to demonstrate that the granulocyte-derived prokineticins may be major determinants in triggering inflammatory pain and PKRs may represent a therapeutic target for the development of novel peripherally acting antinociceptive drugs. First, we demonstrate, in animal models of inflammatory pain, that PK2 and PK2L expression levels timely correlate with the beginning and development of pain behavior. We identified the inflammatory cells that synthesize PK2 proteins and investigated whether PK2 protein released by these cells can produce hyperalgesia. Then, using *pkr1*- and *pkr2*-null mice, we evaluated the role of PKRs in modulating PK2 expression levels and inflammatory hyperalgesia. Finally, we studied the antinociceptive activity of a selective nonpeptide PKR antagonist (23) in acute inflammatory pain.

Results

PK2 Expression Levels Timely Correlate with Development and Duration of Inflammatory Pain. Intraplantar (ipl) injection of complete Freund's adjuvant (CFA) produces an inflammatory reaction with a concomitant swelling of the paw and hypernociception. In both rats and mice the hyperalgesia was already evident 6 h after injection, peaked at 24 h, and recovered to baseline levels in 72 h in mice, but lasted longer in rats. Significant edema formation occurred at the sixth hour. Swelling was maximal after \approx 24 h, then began to decrease. Paw volume returned toward normal values in \approx 3 days in mice, but in 1 week or more in rats. In both rats and mice PK2 mRNA expression levels in the paw tissue correlate with the time course of the CFA-induced hyperalgesia (Fig. 1). RT-PCR analysis performed on rat pad skin (Fig. 2A), collected at several time points after saline or CFA injection, revealed that expression levels of PK2 and PK2L in saline-injected paws were about equally low. In CFA-injected paws PK2 and PK2L expression strongly increased, reaching levels several hundred-fold higher than in the contralateral saline-injected paw

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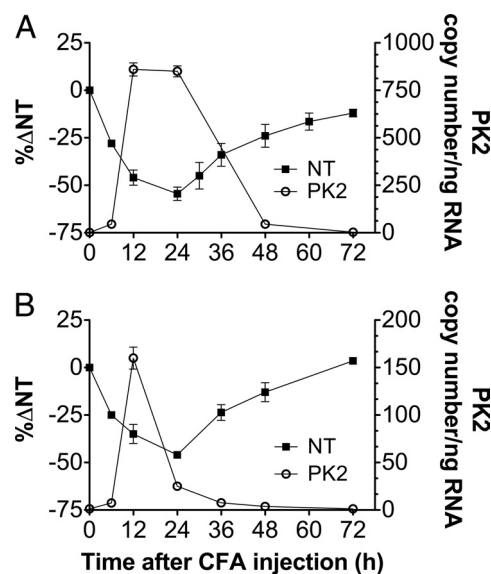


Fig. 1. Correlation between the time course of PK2 mRNA expression in inflamed paws (right y axis) and hyperalgesia (left y axis) after ipl injection of CFA in rats (A) and mice (B).

(900 ± 105 copy number per ng RNA vs. 1 ± 1 copy number per ng RNA). PK2 expression was maximal from 9 to 24 h then began to decrease. At 72 h, it was still ≈10 times higher than in the control paw. PK2L peaked at 9 h and returned to normal levels in 72 h. In mice (Fig. 2B), in saline- and CFA-injected paws the expression levels of PK2L were significantly lower than those of PK2 (0.02 ± 0.01 and 1.3 ± 1 copy number per ng RNA vs. 1.8 ± 1 and 160 ± 25 copy number per ng RNA). PK2 was strongly

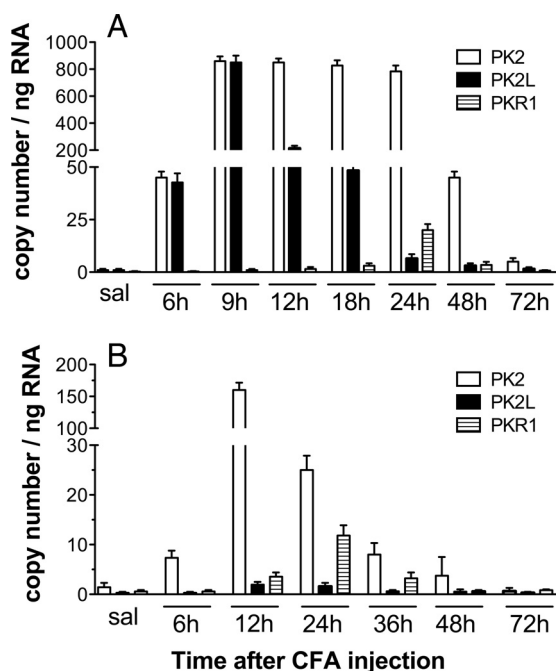


Fig. 2. Time course of PK2, PK2L, and PKR1 expression in rat and mouse paw tissue after ipl CFA, evaluated by real-time RT-PCR. (A) In rats expression levels of PK2 and PK2L were comparable. (B) In mice the expression levels of PK2L were significantly lower than those of PK2. In both rats and mice PKR1 transcripts peaked 24 h after CFA injection. Results are presented as input copy number of the gene of interest per ng of total RNA.

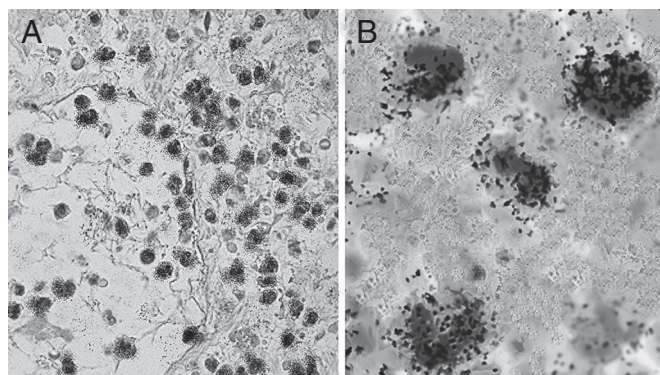


Fig. 3. Autoradiographic images of inflamed mouse paw section (12 h after CFA) hybridized with ³⁵S-PK2 antisense riboprobe and counterstained with toluidine blue–light green solution. (A) PK2 riboprobe revealed PK2 mRNA (black granules) in PMNs along the capillary wall and into extravascular space of the derma (40×). (B) Higher magnification (100×) of PK2-expressing granulocytes.

up-regulated by CFA injection, reaching at 12 h an expression level 120-fold higher than in contralateral saline-injected paw. In both rats and mice PKR1 transcripts peaked 24 h after CFA injection, reaching levels 15- to 30-fold higher than those in the contralateral noninflamed paw (20 ± 3 vs. 0.6 ± 0.5 copy number per ng RNA in rats; 12.5 ± 3 vs. 0.65 ± 0.5 copy number per ng RNA in mice). PK1 and PKR2 transcript levels were very low and did not change after CFA injection.

In situ hybridization with radiolabeled PK2 riboprobes was performed on sections of mouse paw 12 h after CFA or saline injection. Strong signal was evident only in CFA-injected paws, mainly around vessels, which probably was associated with infiltrating cells (Fig. 3).

PK2 and PKR1 mRNA in Cells Sorted from CFA-Injected Mouse Paw. To identify the cells expressing PK2 and quantitate transcript levels of PK2 and PKR1 we prepared cell suspensions from mouse paws collected 12 and 24 h after CFA or saline injection. To obtain enough material we pooled inflamed paws of 20 mice for each point. Flow cytometric analysis indicated that neutrophils (Mac-1+Gr-1+) were more abundant than macrophages (Mac-1+Gr-1-) in the paws collected 12 h after CFA (18 ± 3% and 9 ± 3% of total isolated cells, respectively), but macrophages became as abundant as neutrophils 24 h after CFA injection (25 ± 4% and 22 ± 5%, respectively). CD3+ lymphocytes were undetectable. There were no detectable infiltrating cells in noninflamed paw; hence we used neutrophils and macrophages obtained from the spleen of control mice as a model of nonactivated immune cells. RT-PCR analysis performed on neutrophils and macrophages sorted from spleen of control mice demonstrated that neutrophils are the major source of PK2 mRNA (PK2 copy number per ng RNA: 23 in neutrophils vs. 3 in macrophages). The paw inflammation induced a marked PK2 up-regulation in both spleen (2,500 copy number per ng RNA) and paw (2,060 copy number per ng RNA) neutrophils. A substantially lower up-regulation (8- to 10-fold) was detected in macrophages (Fig. 4A). PKR1 transcript levels were comparable in neutrophils and macrophages from spleen of control mice. The inflammation did not change PKR1 levels in neutrophils but increased it ≈3 times in activated macrophages (Fig. 4B).

Granulocyte-Derived PK2 (GrPK2) Induces Hyperalgesia. In rats, i.p. injection of oyster glycogen induced marked extravasation of granulocytes into the peritoneal cavity and decreased the systemic nociceptive threshold as assessed with the paw-pressure test. Hyperalgesia started 2 h after i.p. injection was maximal

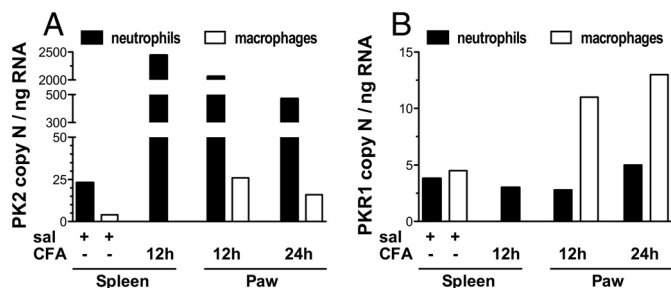


Fig. 4. PK2 (A) and PKR1 (B) expression in immune cells purified by FACS from spleen of saline- or 12-h CFA-injected mice, and from paws of 12- and 24-h CFA-injected mice. Inflammation dramatically increased PK2 expression in neutrophils and slightly increased PKR1 expression in macrophages. PKR1 expression was 3-fold higher with respect to control cells. Results are presented as input copy number of the gene of interest per ng of total RNA.

from 6 to 9 h, then decreased showing normal values 12 h after i.p. injection (see Fig. 8F). Control rats, i.p.-injected with saline, did not show any changes in the paw-pressure threshold over a 9-h observation period. RT-PCR analysis demonstrated that extravasated peritoneal granulocytes, collected 6 h after glycogen injection, express PK2 mRNA at levels enormously higher ($10,500 \pm 900$ copy number per ng mRNA) than the peritoneal granulocytes collected 6 h after saline injections (2.3 ± 2 copy number per ng mRNA) (Fig. 5A). Gel electrophoresis of RT-PCR amplification products demonstrated that oyster glycogen-elicited peritoneal granulocytes express both PK2 isoforms, the canonical PK2 similar to the amphibian Bv8 and the long form, PK2L, already described in mouse and human testis (6, 22). Strong signal corresponding to PKR1 mRNA is also evident (Fig. 5B). 2D electrophoresis of peritoneal granulocyte proteins revealed a spot of ≈ 10 kDa and 8.9 pI that subsequent purification

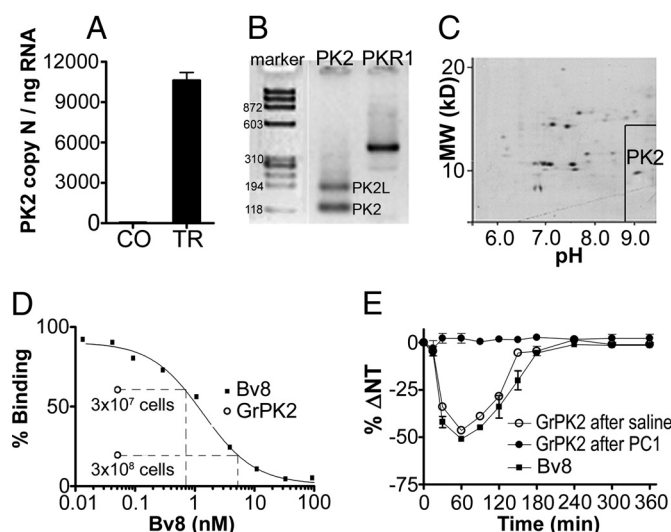


Fig. 5. Purification of GrPK2. (A) PK2 mRNA expression levels in peritoneal granulocytes collected 6 h after i.p. injection of saline (CO) or oyster glycogen (TR). (B) Gel electrophoresis of PK2, PK2L, and PKR1 transcripts, amplified by RT-PCR, in oyster glycogen-elicited peritoneal granulocytes. (C) 2D electrophoresis of peritoneal granulocyte proteins revealed a spot of ≈ 10 kDa and 8.9 pI. (D) Displacement curve of 125 I-MIT from membrane preparations of PKR1-transfected CHO cells by graded concentrations of Bv8. Granulocyte extract (step C fractions) corresponding to 3×10^7 and 3×10^8 cells displaced 125 I-MIT as ≈ 0.7 and 6 nM Bv8, respectively. (E) Time course of hyperalgesia induced in rats by ipl injection of Bv8 (0.5 ng) or GrPK2 corresponding to 3×10^6 cell lysate after pretreatment with saline or the PKR1-preferring antagonist PC1 (0.01 μ g/ipl).

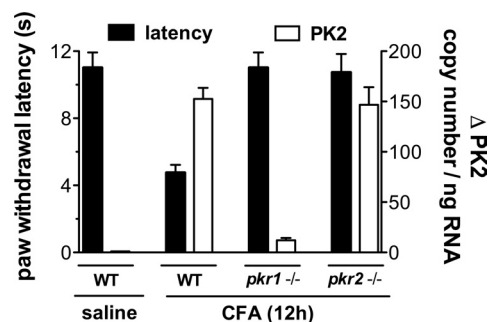


Fig. 6. In WT mice CFA-induced paw inflammation reduced the paw withdrawal latency (left y axis, paw immersion test, 48 °C) with respect to saline-injected paw (4 vs. 12 s). Withdrawal latency of inflamed paw was significantly longer in *pkr1*- and *pkr2*-null mice than in WT mice, whereas inflammation-induced PK2 up-regulation (right y axis) was significantly lower in *pkr1*-null mice than in WT and *pkr2*-null mice.

steps and biological tests allowed us to identify as authentic rat PK2 (Fig. 5C). We did not identify any PK2L-like protein. The PK2 protein purified from rat peritoneal granulocytes (GrPK2) concentration-dependently displaced 125 I-MIT from membrane preparation of CHO cells stably transfected with the PKR1 (Fig. 5D) and, when injected ipl in rats, produced Bv8-like hyperalgesia that was antagonized by prior administration of the PKR1-preferring antagonist PC1 (0.01 μ g/ipl) (Fig. 5E). Starting with 2×10^9 peritoneal granulocytes, the estimated yield in Bv8-like protein was ≈ 320 ng. Analysis of the N-terminal fragment (PRIMM) revealed the amino acid sequence AVITGACDKD that perfectly matches that of rat PK2 (Swiss Protein Bank accession no. NML138852).

Involvement of the PKRs in Inflammation-Induced Hyperalgesia and PK2 Up-Regulation. In WT mice CFA-induced inflammation reduced paw withdrawal latency to the noxious thermal stimulus and greatly up-regulated PK2 mRNA with respect to noninflamed paw (saline). In comparison with WT-mice, *pkr1* (15) or *pkr2* gene disruption significantly reduced CFA-induced inflammatory hypersensitivity, but only the *pkr1* gene disruption reduced inflammatory PK2 mRNA up-regulation. Indeed, PK2 transcript levels 12 h after CFA injection were 15-fold lower in *pkr1*-null mice than in WT mice, whereas PK2 transcript levels in *pkr2*-null mice were similar to those in WT mice. These results indicate that, although both PKRs modulate inflammatory pain, PKR1 alone is involved in modulating in *pkr1*-null mice PK2 expression (Fig. 6).

PKR Antagonism Is a Novel Pharmacological Strategy for the Therapy of Inflammatory Pain. The nonpeptide PKR1-preferring antagonist PC1 (23), preinjected (0.01 μ g per paw) into paws of mice (Fig. 7), antagonized the hyperalgesic effect of exogenous Bv8 (0.5 ng per paw) but was ineffective against other proalgesic mediators such as bradykinin (2 μ g per paw), prostaglandin E2 (PGE2) (1 μ g per paw), and 2-methylthioATP (100 nmol per paw) (Fig. 7). PC1 local (0.01–0.05 μ g per paw) and systemic (up to 150 μ g/kg) injection did not change basal thermal and mechanical pain threshold in rats and mice. Moreover, in rats and mice, PC1 antagonized CFA-induced inflammatory pain. In mice, ipl injection of 0.01 μ g of PC1 abolished the inflammation-induced thermal hypernociception (Fig. 8A) and 0.05 μ g of PC1 ipl. abolished the reduction in the body weight-bearing of the inflamed paw as assessed by the incapacitance test (Fig. 8B) for ≈ 1 h. Indeed, CFA-induced inflammation caused a significant reduction in body weight distribution (ratio = 0.6) across the hind limbs, as the mice placed less weight through the ipsilateral limb. PC1 (0.05 μ g, ipl) allowed

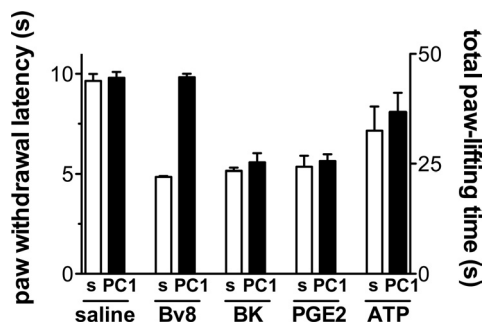


Fig. 7. Ipl injection of PC1 (0.05 μ g) did not change the basal thermal nociceptive threshold of mice (paw-immersion test, 48 $^{\circ}$ C). Pretreatment with PC1 (0.01 μ g, ipl) abolished the thermal hyperalgesia induced by Bv8 (0.5 ng, ipl), but was ineffective in antagonizing the hyperalgesia induced by ipl administration of BK (2 μ g) and PGE2 (1 μ g) (left y axis) and in reducing the paw-lifting time caused by ipl injection of ATP (100 nmol) (right y axis).

the animals to distribute their body weight equally across both hind limbs (ratio = 1, as control mice).

In rats, paw injection of 0.01 μ g of PC1 abolished the inflammation-induced mechanical hypernociception for \approx 2 h. The nociceptive threshold of the inflamed paw rapidly increased, reaching values near those of the contralateral noninflamed paw in \approx 15 min (Fig. 8C). The longer duration of effect of PC1 in mechanical hyperalgesia in rats (Fig. 8C) compared with the effect in thermal hyperalgesia in mice is probably caused by different pharmacokinetics in the two species. Indeed increasing the PC1 doses in mice (0.05–0.2 μ g ipl) prolongs the antihyperalgesic effect up to 2–3 h (Fig. 8A). Also by s.c. injection, PC1 (20, 50, and 150 μ g/kg) raised the pressure pain threshold of the inflamed paw toward the threshold value of the control paw. The antihyperalgesic effect was dose-dependent and lasted for \approx 2 h (Fig. 8D). Repeated systemic administration of PC1 (150 μ g/kg at 6 and 24 h after CFA) also reduced the paw edema and accelerated the recovery to normal paw volume after the insult. The PK2 expression levels, assessed 2 days after CFA injection, were significantly lower (4-fold) in the

inflamed paws of PC1-treated rats than in the inflamed paws of saline-treated rats (Fig. 8E). PC1 (150 μ g/kg, s.c.) abolished the systemic mechanical hyperalgesia caused by oyster glycogen-induced peritonitis (Fig. 8F).

Discussion

We have previously demonstrated that systemic or local injections of very low doses (in the range of fmol) of the amphibian prokineticin Bv8 decrease nociceptive thresholds to thermal, mechanical, and chemical stimuli by activating PKR1 and PKR2 in the primary sensory neurons. This increase in nociceptor excitability resulted from functional cooperation between PKR1 and TRPV1, the two receptors being coexpressed in small sensory neurons of DRG (14–16).

Leukocytes of myeloid origin are a major source of endogenous mammalian Bv8, i.e., the PK2. Bv8/PK2 is overexpressed in inflamed tissue associated to infiltrating cells, hence Bv8/PK2 could be a link between polymorphonuclear cells (PMN) infiltrating the inflamed tissue and the development of inflammatory pain. Here, we demonstrate that the development and duration of CFA-induced hyperalgesia correlates with the expression level of PK2 in the inflamed paw, granulocytes infiltrating the tissue are the inflammatory cells overexpressing PK2, and the PK2 protein, isolated from peritoneal granulocytes, produces Bv8-like hyperalgesia by selectively binding the PKRs. We also demonstrate that blocking the PKRs by a nonpeptide selective PKR antagonist abolishes hypernociception in inflammatory models as well as the deletion of either PKR gene does.

The inflammation produced by paw injection of CFA was of higher intensity and longer duration in rats than in mice, mirroring the increase in PK2 expression that was 10 times higher in rats than in mice and lasted 12 h longer in rats. Granulocytes are the major source of PK2 in the inflamed paw skin; moreover the granulocytes sorted from the mouse paw 12 h after CFA injection are richer in PK2 mRNA than those sorted after 24 h (Fig. 4A), demonstrating a time dependency in CFA-induced up-regulation of PK2 in granulocytes. Nevertheless, granulocytes sorted from the spleen of inflamed mice showed elevated PK2 expression levels like those ob-

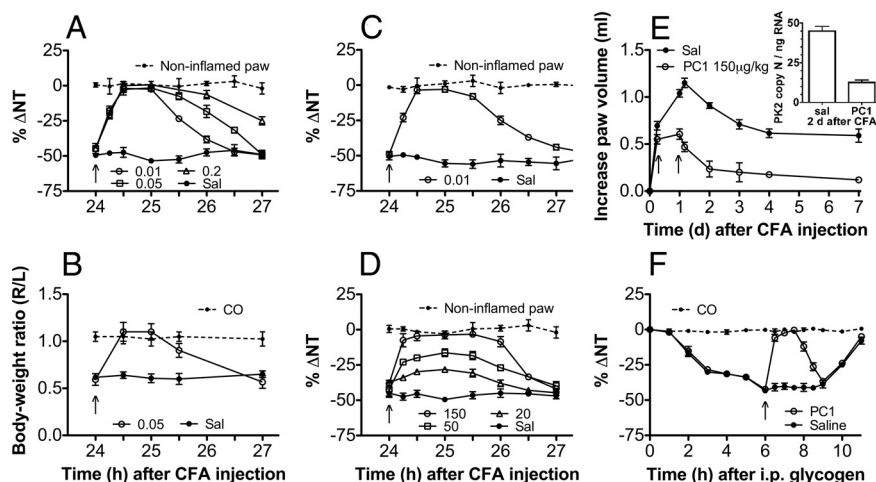


Fig. 8. Antihyperalgesic and antinflammatory effect of ipl and s.c. injection of the PKR1-preferring antagonist PC1 in mice and rats. (A and B) In mice ipl injection of PC1 24 h after CFA reverted the thermal hyperalgesia (0.01, 0.05, and 0.2 μ g) for 1–3 h (A) and abolished the CFA-induced reduction in the body weight-bearing of the inflamed paw (0.05 μ g) for \approx 1 h (B). (C) In rats, PC1 (0.01 μ g, ipl) reverted CFA-induced mechanical hyperalgesia for \approx 2 h. (D) In rats, PC1 dose-dependently antagonized the CFA-induced hyperalgesia also by systemic injections (20–150 μ g/kg, s.c.), bringing the nociceptive threshold of inflamed paw toward that of contralateral noninflamed paw. (E) In rats, repeated administration of PC1 (150 μ g/kg, s.c.), at 6 and 24 h after CFA injection, accelerated recovery of inflamed paw to normal paw volume and significantly reduced the inflammation-induced PK2 up-regulation (Inset). In rats, i.p. injection of oyster glycogen induced marked extravasation of granulocytes into peritoneal cavity and decreased the nociceptive threshold evaluated with the paw-pressure test. PC1 (150 μ g/kg, s.c.) abolished the systemic hyperalgesia caused by GrPK2, released into circulation. (F) Intraperitoneal injection of saline did not modify the mechanical nociceptive threshold (CO).

Flow Cytometry and Cell Sorting. Freshly isolated cells from mouse spleen were prepared and stained as described (33). Paw tissue samples were harvested 12 and 24 h after injection of CFA to obtain a single cell suspension as reported (34). Cells were counted and analyzed on a FACSCalibur with CellQuest software (BD Biosciences). PE-anti-Gr-1 (RB6-8C5) and FITC-anti-Mac-1 (M1/70) antibodies were purchased from BD Biosciences. For cell sorting, cells were appropriately stained, and Mac-1+Gr-1+ and Mac-1+Gr-1- populations were purified (purity $\geq 95\%$) with a FACSARIA sorter (BD Biosciences).

Purification and Isolation of Prokineticins from Peritoneal Granulocytes. Peritonitis was induced in male Sprague-Dawley rats ($n = 100$) by i.p. administration of oyster glycogen (1% in PBS), and the extravasated cells were collected 6 h later (35). The purity of granulocytes, as assessed by Wright-stained smears, was $>95\%$. Harvested cells were washed, centrifuged at $4,000 \times g$ at 4°C , resuspended, and lysed with 2D Protein Extraction Buffer I (GE). After centrifugation, an aliquot of the supernatant ($\approx 1/20$ volume) was submitted to 2D electrophoresis to separate granulocyte proteins, and the remaining supernatant was added to 4 volumes of 0.2% TFA in acetonitrile/isopropanol (4:1). The extract was centrifuged at $4,000 \times g$ for 10 min at 4°C , and the supernatant was collected and stored at -80°C (step A).

First-dimension isoelectric focusing was performed by means of Bio-Rad Protean IEF Cell using 17-cm Bio-Rad ReadyStrip IPG strips, pH 3–10. Second-dimension SDS/PAGE was performed by means of Bio-Rad PROTEAN II XL cell using the Protean II Ready Gel precast gels in Tris/glycine/SDS running buffer, pH 8.5. In SDS/PAGE electrophoretic gels protein spots were revealed with the silver-staining method.

The extract (step A) was lyophilized, dissolved in 20 mM sodium phosphate buffer (pH 6.4), loaded on cationic exchanger Source 30S column (GE), and eluted by a gradient 0–100% of 0.4 M NaCl in 20 mM sodium phosphate buffer, pH 6.4 (step B). Eluates containing hyperalgesic activity (from 25 to 35% of 0.4 M NaCl) were reunited and further purified by gel filtration [Superdex 75 10/300 column (GE), eluting buffer 1 M acetic acid, 1 mL/min, UV detection 220 and 280 nm] to eliminate salts that interfere with binding assay (step C). Collected fractions were assayed, in vivo, for the presence of Bv8-like hyperalgesic activity and, in vitro, for PKR1 binding assay on PKR1-transfected CHO cells. The fractions containing bioactivity were reunited, lyophilized, dissolved in 0.2% TFA in water, loaded on a reversed-phase C8 column (Vydac), and eluted by a gradient 15–100% acetonitrile containing 0.2% TFA (step D). The biological active fraction (PKR1 binding)

was further chromatographed on a Grace-Vydac 238 EV 51 column before submitting to sequence analysis (PRIMM).

Receptor Binding Assay. The fractions purified by gel filtration (step C) were evaluated for their ability to displace [^{125}I]MIT binding from membrane preparations of PKR1-transfected CHO cells as described (36). The displacement curve of [^{125}I]MIT by graded concentrations of Bv8 (0.01–100 nM) was used to generate a standard curve to determine the content of Bv8-like proteins in each tested fraction (PRISM; GraphPad).

Animals and Hypernociceptive Tests. All experiments were performed under protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health according to European Commission directives. Male Sprague-Dawley rats (250–300 g), C57BL/6 (25–30 g), *pkrl1*($-/-$) and *pkrl1*($+/+$), and *pkrl2*($-/-$) and *pkrl2*($+/+$) mice (Lexicon Genetics) were housed in temperature-controlled rooms (22–25 $^\circ\text{C}$) with access to water and food ad libitum. Pain threshold was evaluated by the paw-pressure test in rats and the paw-immersion-in-hot-water test (48 $^\circ\text{C}$) in mice as described (14, 15). Mice were tested for changes in mechanical hypersensitivity by using a body weight bearing averager (Incapacitance Tester; Biological Instruments). Results were expressed as mean ipsilateral/contralateral ratio. The data are presented as mean \pm SE mean values.

Inflammatory Pain Models. Peritonitis was induced in rats by i.p. administration of oyster glycogen as described above. Paw inflammation was induced by ipl injection of CFA (Sigma) in mice (20 μL per paw) and rats (100 μL per paw). Paw volume was measured with a 7140 plethysmometer (Ugo Basile).

Drug Injections. Drugs were injected topically into the plantar region of hindpaws (ipl), in volumes of 20 μL in mice and 100 μL in rats and s.c. into the flank region in a volume of 2 mL/kg. GRPK2 (isolated from peritoneal granulocytes) and amphibian Bv8 (purified from skin secretion of *B. variegata*) (5) were injected ipl in mice and rats. PC1 synthesized by Balboni et al. (23) was injected ipl and s.c. in mice and rats. Bradykinin (Sigma-Aldrich), PGE2 (Sigma-Aldrich), and 2-methylthioATP (Tocris) were injected ipl in mice.

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